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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

O'Hagan *et al.* Confirmation No.: 7716

Serial No.: 09/724,661 Art Unit: 1648

Filing Date: November 28, 2000 Examiner: STUCKER, J.

Title: USE OF HYALURONIC ACID POLYMERS FOR MUCOSAL DELIVERY OF VACCINE ANTIGENS

DECLARATION OF DEREK O'HAGAN, Ph.D.

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, Derek O'Hagan, hereby declare as follows:

1. I received my Ph.D in Pharmaceutical Sciences from the University of Nottingham, UK in 1987 and focused my studies on pharmaceutical formulations as immunological adjuvants and specifically studied microparticles for vaccine delivery.

2. I have been employed by Chiron Corporation since 1995 and I am currently the Senior Director of Vaccine Research and the Head of Delivery Systems and Adjuvants. My principal responsibility is to direct and manage a research program concerned with the design, preparation, characterization and evaluation of vaccine adjuvants and delivery systems. Programs include vaccines against viral and bacterial pathogens, and include vaccines for systemic and mucosal administration of proteins, protein/polysaccharide conjugates and DNA. I am therefore extremely familiar with particulate vaccine delivery systems, having actively studied and worked in this discipline for over 20 years. I have coauthored numerous publications and patents in the field of vaccine delivery, including publications relating to mucosal administration of vaccine antigens. A copy of my Curriculum Vitae is attached hereto as Exhibit A.

3. I have reviewed relevant documents from the prosecution of the above-referenced application (hereinafter "the application") on which I am a coinventor,

including the Office Action dated May 14, 2003 and the art cited therein. I understand the claims have been rejected over the combination of EPA 517,565 to Callegaro et al. ("Callegaro") in view of Partidos et al., *Immunology* (1996) 89:483-487 ("Partidos") and JPO 05163161 to Koichiro et al. ("Koichiro"). I understand that the claims pending in the application are directed to compositions comprising a hyaluronic acid ester polymer in the form of a microsphere, an antigen and an adjuvant. The pending claims also relate to methods of making compositions comprising the microspheres, as well as methods of mucosal administration of the compositions. The compositions are extremely useful for mucosal delivery.

4. I do not agree the above combination describes the invention claimed or renders the claimed invention obvious, i.e., that the differences between the invention as claimed and the subject matter of the cited art are such that they would be obvious to one skilled in the art, such as myself, as of the filing date of the patent application or earlier. My opinion is based on the facts set forth below, my familiarity with the subject matter, and particularly derives from the surprising and unexpected finding that the hyaluronic acid microspheres in combination with a coadministered adjuvant is able to stimulate exceptionally potent immune responses at levels far above those responses stimulated by the delivery of antigen alone, or delivery of antigen with a hyaluronic acid microsphere alone.

5. In particular, Callegaro relates to the delivery of "biologically active molecules" using an ester of hyaluronic acid or mixtures of the esters. The biological molecules delivered by Callegaro are therapeutic in nature, that is, they are provided in order to treat a disease or disorder. They are not administered in order elicit an immune response. In fact, such a response is **highly undesirable** and considered an unsafe side-effect in the therapeutic context. Indeed, this is one of the criteria the FDA examines in order to determine whether a drug is safe prior to approval. Thus, I would not be led to use hyaluronic acid microspheres for delivery of an antigen upon a reading of Callegaro. Rather, I would be led to believe that hyaluronic acid microspheres would be ineffective for eliciting an immune response and would therefore be ineffective in a vaccine context.

6. Partidos does not relate to the use of microparticles, hyaluronic or

otherwise, for delivery of vaccine antigens. Rather, Partidos coadministers the LTK63 mutant with an antigen that is not associated with another delivery system. Partidos does not speak to the use of additional adjuvants or systems in combination with the LTK63 mutant. Since Callegaro does not relate to delivery of vaccine antigens, but rather therapeutics, I would not combine hyaluronic acid particles as described in Callegaro with the LTK63 mutant described in Partidos, to arrive at a system that provides such enhanced immunogenicity to an antigen delivered as claimed.

7. Finally, the Koichiro abstract describes delivering an antigen with hyaluronic acid. However, Koichiro does not appear to administer an additional adjuvant with the vaccine. Contrary to Koichiro's report, we have found that a system using hyaluronic microspheres alone with an influenza antigen, without coadministered adjuvant, is ineffective at producing an immune response. In particular, appended as Exhibit B is Singh et al., *J. Control. Rel.* (2001) 70:267-276, on which I am a coauthor. As seen in Figures 1 and 2 of Singh et al., the combination of the influenza antigen (HA) with a hyaluronic microsphere and a coadministered adjuvant was exceptionally potent, while use of antigen with the hyaluronic microsphere alone did not elicit a significant immune response. In fact, the response elicited using intranasal delivery of the antigen, hyaluronic microsphere and adjuvant was so potent that it was actually better than injection of the same vaccine in rabbit (Figure 4) and sheep (Figures 5 and 6). This discovery is consistent with the Callegaro reference which, as explained above, describes a delivery system for therapeutic proteins where an immune response is not desired.

8. Based on the foregoing, it is my opinion that a skilled scientist working in the field of antigen delivery would not have considered delivering vaccine antigens from using hyaluronic acid microspheres as claimed from a reading of the combination of Callegaro, Partidos and Koichiro, and certainly would not have expected to see such increased antibody titers using the system as claimed.

9. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 6/11/04 Signature: Derek O'Hagan

Derek O'Hagan, Ph.D.

NAME: Derek Thomas O'Hagan.

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EDUCATION: Bachelor of Pharmacy with Honours; Member of Royal Pharmaceutical Society of Great Britain, PhD in Pharmaceutical Sciences.

EXPERTISE: Vaccine delivery systems; immunological adjuvants; controlled release technologies; formulation science; delivery technologies; microencapsulation; drug delivery; mucosal vaccines.

WORK EXPERIENCE.

2003 – Head of Delivery Systems and Adjuvants, Chiron Vaccines.

2003 – Senior Director, Vaccine Research, Chiron Corp.

2000 - Director, Vaccine Adjuvants and Delivery Systems, Chiron Corporation, Emeryville, CA.

1998 - Associate Director, Vaccine Research, Chiron Corp.

1995 - Senior Scientist, Adjuvant Research, Chiron Corp.

My principal responsibility is to direct and manage a research program concerned with the design, preparation, characterization and evaluation of vaccine adjuvants and delivery systems. Programs include vaccines against viral and bacterial pathogens, and include vaccines for systemic and mucosal administration of proteins, protein/polysaccharide conjugates and DNA. Several novel approaches have been invented by my group and some been moved into human clinical trials.

In addition, I am a member of the Vaccines Research Council in Chiron, with oversight and responsibility for all vaccine research activities and programs. Additional responsibilities include:

- Direction of adjuvant discovery research program.
- Initiation and management of external scientific collaborations.
- Interaction with Process Development to ensure effective transfer of delivery technologies.
- Interaction with internal and external attorneys on the filing and defence of intellectual property.
-Interaction with senior colleagues to define future research directions and long range plans.
-Interaction with commercial colleagues to identify which technologies could result in successful products.

In addition, I have also been responsible for developing novel approaches for the delivery of therapeutic proteins, including microencapsulation and controlled release technologies.

1993 - Director, Vaccine Formulation, United Biomedical, Hauppauge, NY.

Responsibilities included the supervision of a group working on vaccine delivery technologies, mainly for peptide vaccines. In addition, I was responsible for the scale-up, manufacture and quality control of vaccines for clinical evaluation. Main achievements included;

- Submission of a successful IND application to the FDA for an oral HIV vaccine entrapped in microparticles, which was evaluated through the AIDS Vaccine Evaluation Group of the NIH.
- A lipopeptide vaccine, comprising an HIV-1 gag peptide linked to a branched lipid tail, was synthesized, formulated and approved by the FDA as an IND.
- A multicomponent peptide vaccine adsorbed to Alum was also approved as an IND.

1989 - Lecturer in Drug Delivery, Department of Pharmaceutical Sciences, University of Nottingham.

I was responsible for establishing and directing a research group focused on vaccine delivery systems for systemic and mucosal administration, including microencapsulated vaccines.
The main achievements of the group included;

- Research funding was obtained from national and international agencies, including WHO, MRC, NIBSC, The Wellcome Trust, EU and MRC ADP.
- Research funding was also obtained from the pharmaceutical industry, including Smithkline Beecham, Hoechst UK and The British Technology Group.
- A number of key publications which first described and then firmly established the potential of microparticles and polymers for vaccine delivery originated from this group.
- Supervision of five Ph.D. students and five Post-Doctoral Research Fellows.

1988 - Post-Doctoral Research Fellow, Department of Pharmaceutical Sciences, Nottingham.
Microparticles for the development of a HIV vaccine - funded by the MRC ADP.

Main achievements included;

- Established techniques for the preparation of microparticles with entrapped antigens.
- Demonstrated the uptake of microparticles into the M cells of Peyer's patches.
- Established a number of diverse external collaborations to allow a thorough evaluation of the potential of microparticles as vaccines.

1987 - Research Scientist, Danbiosyst UK Ltd., Nottingham.
Nasal delivery of biosynthetic human growth hormone (hGH) - funded by Novo-Nordisk, Denmark.

Main responsibilities included the establishment of a number of techniques to investigate the structural integrity of human growth hormone (hGH) following formulation with intranasal absorption enhancers. The techniques established included GPC, IE-HPLC, PAGE and IEF. In addition, I developed ELISA to quantify the levels of hGH *in vivo* following intranasal delivery in several animal models.

EDUCATION:

1984 – 87, Ph.D. Pharmaceutical Sciences, University of Nottingham, UK.
'Pharmaceutical formulations as immunological adjuvants' - Sponsored by the Science and Engineering Research Council and Beecham Pharmaceuticals, Surrey. Supervisors; Prof. S.S.Davis and Dr. K. Palin.

The topic of my thesis, 'microparticles for vaccine delivery' was my own suggestion and was a new area of research for the department and for my supervisors. Therefore, I was required to establish a number of techniques, including antigen encapsulation technologies, ELISA, RIA, PAGE and WB. These

techniques were used to evaluate antigen integrity following encapsulation in microparticles and liposomes and to evaluate immune responses following immunization of small animal models.

1983 - Pre-registration Pharmacist, Walton Hospital, Liverpool.

Qualified as a pharmacist and a member of The Royal Pharmaceutical Society of Great Britain in 1984.

1980 - Bachelor of Pharmacy, University of Nottingham, UK.

Bachelor of Pharmacy with Honours, Second Class, Division 1.

AWARDS AND HONOURS:

Controlled Release Society,
- Board of Scientific Advisors, 2000.

Controlled Release Society,
- Research Achievement Award, 1999.

Royal Pharmaceutical Society of Great Britain.
- Conference Science Medal Award, 1997.

Pharmaceutical Research
- Editorial Board, 2001.

CRC Critical Reviews in Therapeutic Drug Carrier Systems
-Editorial Board, 1998.

Vaccine
- Editorial Board, 1996.

Vaccine, Adjuvant Research
- Editorial Board, 1995.

Publications includes >70 research papers, 12 reviews, 15 book chapters, 2 books edited and named inventor on >50 patents. Full list available on request.

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A novel bioadhesive intranasal delivery system for inactivated influenza vaccines

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Abstract

The aim of the current studies was to evaluate a bioadhesive delivery system for intranasal administration of a flu vaccine, in combination with a mucosal adjuvant (LTK63). A commercially available influenza vaccine, containing hemagglutinin (HA) from influenza/A Johannesberg H1N1 1996, and LTK63 or LTR72 adjuvants, which are genetically detoxified derivatives of heat labile enterotoxin from *Escherichia coli*, were administered IN in a bioadhesive delivery system, which comprised esterified hyaluronic acid (HYAFF) microspheres, to mice, rabbits and micro-pigs at days 0 and 28. For comparison, additional groups of animals were immunized intranasally with the HA vaccine alone, with soluble HA+LTK63, or IM with HA. In all three species, the groups of animals receiving IN immunization with the bioadhesive microsphere formulations, including LT mutants, showed significantly enhanced serum IgG responses ($P<0.05$) and higher hemagglutination inhibition (HI) titers in comparison to the other groups. In addition, the bioadhesive formulation also showed a significantly enhanced nasal wash IgA response ($P<0.05$). Most encouragingly, in pigs, the bioadhesive microsphere vaccine delivery system induced serum immune responses following IN immunization, which were significantly more potent than those induced by traditional IM immunization at the same vaccine dose ($P<0.05$).

Keywords: Bioadhesive; HYAFF microparticles; Flu antigen; Immunogenicity

1. Introduction

Since the majority of pathogens initially infect their hosts through mucosal surfaces, the induction of mucosal immunity is likely to make an important contribution to protective immunity. In addition, mucosal administration, which avoids the use of needles, is becoming an increasingly attractive approach for the development of new generation vaccines. Although a number of vaccines are commercially available which control the spread of influenza [1–5], these vaccines induce serum immunity, but do not induce mucosal immunity at the site of infection in the nasal cavity. In addition, commercially available vaccines are ineffective for the induction of cytotoxic T lymphocyte (CTL) responses, which are responsible for killing virally infected cells. Therefore, the currently available vaccines are not considered to be optimal [6,7]. As a consequence, work is currently underway to develop more effective influenza vaccines that induce mucos-

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al IgA responses through local administration, and also induce more potent systemic responses [8–10]. An early study in mice showed that IN immunization with a potent adjuvant induced superior cross-protective immunity than parenteral immunization, supporting the use of the IN route for flu vaccine development [11]. The most potent mucosal adjuvants which are available for local immunization are heat labile enterotoxin from *Escherichia coli* (LT) and cholera toxin (CT) from *Vibrio cholerae*, and these molecules and their subunits have shown some promise as intranasal adjuvants for flu [9–15]. However, since the native toxins CT and LT are the causative agents, respectively for cholera and traveler's diarrhea, they are considered to be too toxic for use in humans. Therefore, several groups have focused on the development of detoxified mutants of LT and CT as mucosal adjuvants. Colleagues within Chiron have focused on the development of LT mutants with reduced, or eliminated enzymatic activity, since it is the ADP-ribosylating enzymatic activity of LT and CT which causes abnormal intracellular accumulation of cAMP and excess fluid secretion from intestinal cells. We have used site-directed mutagenesis to replace single amino acids within the enzymatic A subunit of LT and have developed mutants (LTK63 and LTR72) with reduced or eliminated enzymatic activity [16]. LTK63 is completely devoid of ADP-ribosyltransferase activity and appears to be non-toxic both in vivo and in vitro, while LTR72 has residual enzymatic activity (<1% of the native LT) and has significantly reduced, but detectable toxicity [16]. Both of these mutants have been previously shown to be potent mucosal adjuvants for antibody and CTL induction in a number of studies in mice [9,15–17].

Since the early 1980s several groups have focused on bioadhesion as a concept to improve local and systemic drug delivery [18,19]. In general, bioadhesive delivery systems are designed to adhere to various tissue surfaces, mainly the mucosal epithelium. An alternative term, mucoadhesion is also used often to describe the interaction of a polymer delivery system and a mucosal site. Mucoadhesion appears to require a highly expanded and hydrated polymer network, which promotes an intimate molecular contact between the delivery system and the mucus layer [20–24]. The mechanisms of bio- or mucoadhesion can involve physical or chemical interactions, including electrostatic or hydrophobic bonding, van der Waal's forces or hydrogen bonding. Irrespective of the mechanisms involved, the main advantages of bioadhesive delivery systems include extended residence time at the site of action, local delivery to a selected site and enhanced interaction with the mucosal epithelium [20]. In a range of studies in recent years, several bioadhesive polymers have been described, including chitosans, methacrylic acids, starch, gelatin, hyaluronic acid and cellulose derivatives to enhance the absorption of co-administered protein drugs [22–25]. Hyaluronic acid is a naturally occurring mucopolysaccharide consisting of residues of D-glucuronic acid and *N*-acetyl-D-glucosamine. Through the esterification of the carboxyl groups of hyaluronic acid with alcohols, biodegradable polymers have been developed, called HYAFF [22]. The HYAFF™ polymers can be used to make microspheres using a coacervation phase-separation process [26]. The HYAFF microspheres have strong bioadhesive properties and have been used for delivery of calcitonin and insulin following mucosal administration [23,24]. However, HYAFF microspheres have not previously been used for mucosal delivery of vaccines.

In the current studies, we report the use of an influenza hemagglutinin (HA) vaccine administered IN to mice, rabbits and micro-pigs along with LT mutants and a bioadhesive delivery system, comprising HYAFF microspheres. The IN route has been used previously to administer vaccines and adjuvants to animal models [8]. The responses to the bioadhesive formulations were compared to HA combined with LT mutants by the IN route, and also HA administered alone by the IN and IM routes. For IM administration, we used a commercially available vaccine which like most other flu vaccines, is unadjuvanted.

2. Materials and methods

2.1. Materials

A monovalent A/Johannesberg split vaccine preparation of purified influenza HA was provided by

Chiron Vaccines, Siena, Italy. Dosing of the vaccine was based on HA content as assayed by single radial immunodiffusion (SRID) as described previously [9]. The mucosal adjuvants, LTK63 and LTR72 were fermented and purified at Chiron, Emeryville, USA. HYAFF microspheres were prepared by a coacervation phase separation technique as previously described [23,24,26] and were provided by Fidia Advanced Biopolymers, Padova, Italy. The bioadhesive properties of HYAFF microspheres has previously been described both in rat [24] and sheep [23] models.

2.2. Methods

The combined bioadhesive vaccine formulations were prepared as follows; HA and LT mutants at the doses described in the text were incubated with HYAFF microspheres in PBS. The suspension was kept at 4°C for 6 h and then freeze dried overnight. Prior to administration to the animal models, the microsphere formulation was re-suspended in PBS, to allow easy administration of the dose as a suspension. The HYAFF dose was 5 mg for mice (25 μl volume of administration) and 20 mg of microspheres per animal for rabbits (200 μl) and pigs (250 μl). The HA and LT mutant doses was changed according to the animal species being tested and details are included in the text.

2.3. Microparticle characterization

The HYAFF microspheres were sized on a Malvern Mastersizer both before and after combination with the antigen and adjuvant. The HYAFF microspheres as provided by the manufacturer had a mean size of 8.4 μm, as previously described [25]. The size following hydration in vitro during association with the the antigen/adjuvant was 32±2.3 μm. After freeze drying, the size of the microspheres in the final formulation was determined to be 8.2±0.6 μm.

2.4. Antigen and adjuvant integrity

Both the HA and the LT mutants were evaluated by an ELISA [9] to evaluate antigenic integrity following formulation with the HYAFF microspheres. The rate of in vitro release of HA and the LT mutants in PBS from the surface of the HYAFF microspheres was also estimated by ELISA [9]

2.5. Immunization protocols

2.5.1. Mice

The first study was a preliminary evaluation in mice of the bioadhesive vaccine delivery system using HYAFF microspheres. The antigen and adjuvant doses used were 10 μg HA and 25 μg LTK63. Four groups of Balb/C mice (10 per group) were immunized IN with either the HYAFF-HA-LTK63 formulation, HYAFF-HA, HA+LTK63 soluble proteins or HA alone. The animals were boosted at day 28 with the same formulations and blood was collected at day 42.

2.5.2. Rabbits

In the first study in rabbits, three groups of New Zealand whites (five or six per group) were immunized IN with either a HYAFF-HA-LTR72 formulation, HA+LTR72 soluble proteins or HA alone. The HA dose in all groups was 25 μg and the LTR72 dose was 50 μg. The animals were boosted at day 28 and blood was collected at days 28 and 42. In a second smaller study, two groups of rabbits (five per group), were immunized IN with a HYAFF-HA-LTK63 combination, with doses of HA at 10 μg and LTK63 at 25 μg. For comparison, a second group of rabbits were immunized IM with HA 25 μg. Blood samples were collected at days 14 and 42.

2.5.3. Micro-pigs

In the pig study, three groups of four Yucatan micro-pigs (8–10 kg) were used and were housed in pairs. In this study, the responses induced by IN immunization were compared to IM immunization. The doses selected for pigs were 25 μg of HA for all groups, and 100 μg of LTK63 for the two IN groups. One group was immunized IN at weeks 0 and 4 with the HYAFF-HA-LTK63 bioadhesive microsphere formulation. A second group was immunized IN with soluble HA+LTK63 at the same dose. For comparison, a third group of pigs were immunized IM with 25 μg HA.

2.6. Immunoassays

Serum total anti-HA IgG were measured by ELISA as previously described [16]. Briefly, ELISA plates (Immulon-1, 96 well, U-bottom, obtained from Dynatech Laboratories, Chantilly, VA) were coated with HA (10 μg/ml) overnight. After blocking (1% goat serum, 0.3% Tween 20 in phosphate-buffered saline), plates were coated with 1:3 serially diluted serum samples. After washing (blocking buffer), the plates were coated with 1:4000 goat anti-pig IgG horseradish peroxidase conjugate (Gibco, Grand Island, NY) and developed using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Absorbances were measured at A_{490} using a standard ELISA reader. The titers represent reciprocal serum dilutions giving an A_{490} of 0.5 and were normalized to a serum standard assayed in parallel.

Nasal wash samples from pigs were assayed for IgA using a bioluminescent immunosorbant assay (BIA) as previously described [9,16]. Briefly, ELISA plates (MicroLite obtained from Dynatech) were first coated with the HA antigen (5 μg/ml) overnight. After blocking (5% goat serum, 25 mM Tris, 10 mM EGTA, 150 mM KCl, 2 mg/ml BSA, 0.3% Tween-20, pH 7.5), plates were coated with 1:3 serially diluted nasal wash samples in blocking buffer. The plates were developed using 1:1000 diluted goat anti-pig IgA biotin conjugate (EY Labs, San Mateo, CA) pre-saturated with purified pig IgG (1 mg/ml, Sigma Chemical Company, St Louis, MO) to reduce IgG cross-reactivity. Plates were then incubated with 1:500 diluted streptavidin–jellyfish aequorin conjugate (SeaLite Sciences, Bogart, GA). Luminescence was triggered with 10 mM calcium acetate and measured using a luminometer (Dynatech ML3000). Quantitation was based on relative light units (RLU) representing total luminescence integrated over 3 s (arbitrary units). Titers represent log dilution values linearly extrapolated from the log RLU data to a cut-off value at least two standard deviations above mean background.

Serum samples for each animal were assayed for hemagglutination inhibition (HI) titers by the Viral and Rickettsial Disease Laboratory (Department of Health Services, Berkeley, CA) using a standard assay based on the ability of sera to inhibit the agglutination of goat red blood cells (RBC) in the presence of HA antigen [28–30]. Fresh RBC were diluted to 0.4% cell suspension using OD_{540} against a cyanmethemoglobin reference standard. A/Johannesberg HA antigen stock was titered to 4 HA units defined as the highest concentration required to agglutinate a 0.2% RBC suspension. Serum samples were serially diluted two-fold into an ELISA plate then the HA antigen at a final concentration of 1 HA unit and 0.2% RBC was added. The HI titer was then defined as the reciprocal dilution of the serum required to completely inhibit agglutination. HI titers are normally determined to reflect the potency of influenza vaccines and these titers have been shown to correlate closely with protective efficacy of vaccines.

2.7. Statistics

Analysis of variance was calculated by using the StatView program for Macintosh computers. Differences among groups of animals at significance levels of 95% were calculated by analysis using Fisher's protected least-significant-difference test.

3. Results

3.1. Antigen and adjuvant integrity and release rates in vitro

The HA antigen and the LT mutants both remained largely intact after formulation and release from HYAFF microspheres in vitro, with no significant changes in antibody binding characteristics (data not shown). The rate of release of both HA and the LT mutants indicated a fairly large burst release, with 32% of LTK63 and 24% of HA released in day 1, followed by a more slow release phase, with 75% of both HA and LTK63 released by day 10 in vitro. We do not consider that the in vitro release rate of the formulations has any relevance for the likely rate of release of antigen and adjuvant in vivo. However, these studies were performed to determine if the antigen and adjuvant were actually released from the formulation and to allow an evaluation of integrity of both following release.

3.2. In vivo immunogenicity

3.2.1. Mice

The group of mice receiving the bioadhesive microsphere formulation (HYAFF-HA-LTK63), showed significantly enhanced serum IgG antibody responses in comparison to the other groups immunized IN with soluble antigen alone, with HA-HYAFF, or with HA+LTK63 ($P<0.05$) (Fig. 1). The HI titers were also highest in the group of mice immunized with the bioadhesive microsphere formulation (Fig. 2). Mouse sera needed to be pooled to obtain sufficient serum to allow the HI titer to be obtained. Therefore, mean values are shown in Fig. 2.

3.2.2. Rabbits

The group of rabbits immunized with the bioadhesive HYAFF-HA-LTR72 formulation had significantly higher serum IgG titers than the groups immunized IN with soluble HA alone, or soluble HA+LTR72 at day 42 ($P<0.05$) (Fig. 3). In addition, in the second rabbit study, the group immunized IN with HYAFF-HA-LTK63 showed a higher mean serum IgG antibody responses than the group immunized IM, although there was no significant difference ($P>0.05$) (Fig. 4). The IN immunized group in this study also showed a clear trend for enhanced HI titers over the IM immunized group (Table 1).

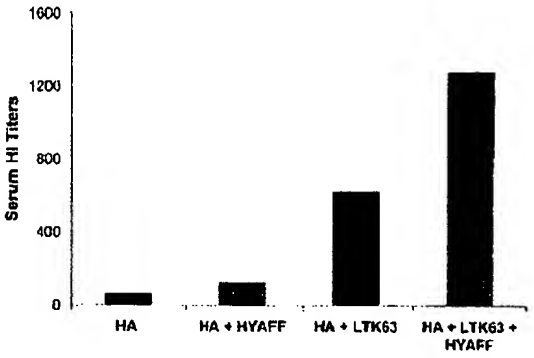


Fig. 2. Serum hemagglutination inhibition titers (HI) in groups of mice ($n=10$) immunized with either HA alone IN, HA+HYAFF IN, HA+LTK63 IN or HA+LTK63+HYAFF IN. The graph shows a single value obtained from pooled sera at day 42.

3.2.3. Micro-pigs

In the pig study, the group receiving the bioadhesive formulation (HYAFF-HA-LTK63) had a significantly higher antibody response than the groups immunized IN with soluble HA+LTK63 and the group immunized IM with HA at day 56 ($P<0.05$) (Fig. 5). The nasal IgA titers were also significantly

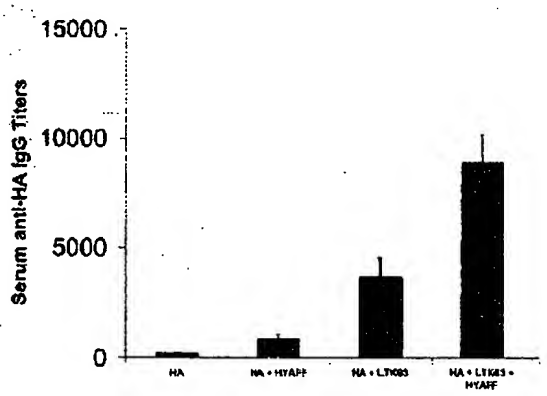


Fig. 1. Anti-HA serum IgG antibody titers in groups of mice ($n=10$) immunized with either HA alone IN, HA+HYAFF IN, HA+LTK63 IN or HA+LTK63+HYAFF IN. The graph represents the geometric mean titers+S.E.M. for each group at day 42. The HYAFF+LTK63+HA formulation was significantly better than all other groups ($P<0.05$).

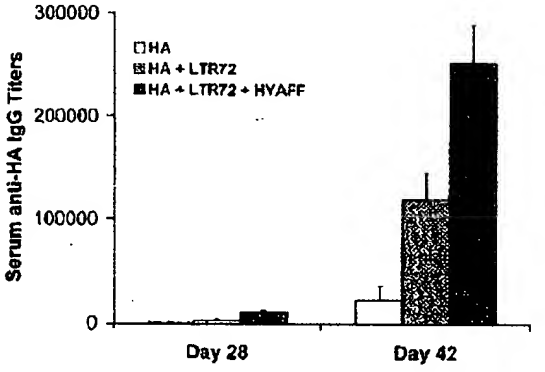


Fig. 3. Serum anti-HA serum IgG antibody titers in groups of New Zealand white rabbits ($n=5$ or 6) immunized with either HA alone IN, HA+R72 IN or HA+R72+HYAFF IN. The graph represents the geometric mean titers±S.E.M. for each group at days 28 and 42. The HYAFF+LTR72+HA formulation was significantly better than soluble HA and HA+LTR72 ($P<0.05$) at day 42.

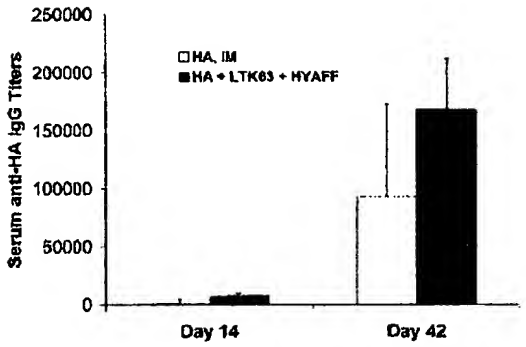


Fig. 4. Anti-HA serum IgG antibody titers in two groups of rabbits (n=5) immunized with either HA alone IM, or HA+LTK63+HYAFF IN. The graph represents the geometric mean titers±S.E.M. for each group at days 14 and 42. The groups were not significantly different from each other at days 14 and 42 (P>0.05).

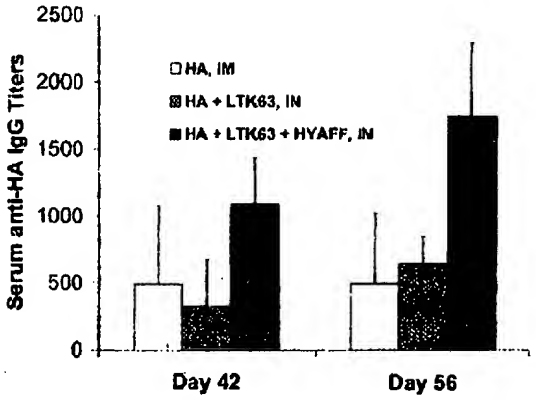


Fig. 5. Anti-HA serum IgG antibody titers in three groups of micro-pigs (n=4 per group) immunized with either HA alone IM, HA+LTK63 IN or HA+LTK63+HYAFF IN. The graph represents the geometric mean titers±S.E.M. for each group at days 42 and 56. The HYAFF+LTK63+HA formulation was significantly better than the HA (IM) and HA+LTK63 (IN) at day 56 (P< 0.05).

higher in the group of animals immunized with bioadhesive microspheres (P<0.05) (Fig. 6). In addition, the HI titers also tended to be higher in the group of animals immunized IN with the bioadhesive microsphere formulation (Table 2).

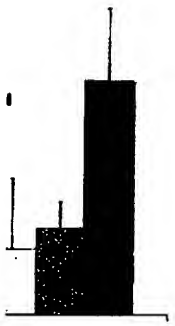
4. Discussion

The initial observations in mice offered significant encouragement that the bioadhesive microsphere delivery system may offer some benefit over administration of soluble HA with the potent adjuvant LTK63. However, the limitations of small animal models and their inability to accurately predict responses in human subjects is well known and widely acknowledged. This is particularly true in relation to mucosal delivery, since mice have very small nasal cavities which can accommodate only very low volumes of fluid (20–25 μl). Therefore, during drug or vaccine delivery studies, the mouse nasal cavity is often completely filled following

Table 1
Hemagglutination-inhibition (HI) titers and serum IgG titers at day 42 from individual animals in two groups of rabbits immunized with either HA alone IM, or HA-LTK63-HYAFF IN

Formulation	Route	Serum IgG ELISA titers (Day 42)	Hemagglutination inhibition titers (Day 42)
HA alone	IM	463112	1280
		77087	160
		68812	320
		28274	640
		99682	160
HA+LTK63+HYAFF	IN	85838	640
		n.d.	n.d.
		255575	1280
		263413	1280
		136493	640

n.d.=not done.



Day 56

in three groups of
either HA alone IM,
N. The graph repre-
ach group at days 42
ion was significantly
(IN) at day 56 ($P<$

potent adjuvant
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curately predict
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commodate only
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idies, the mouse
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Hemagglutination inhibition titers (Day 42)
1280
160
320
640
160
640
n.d.
1280
1280
640

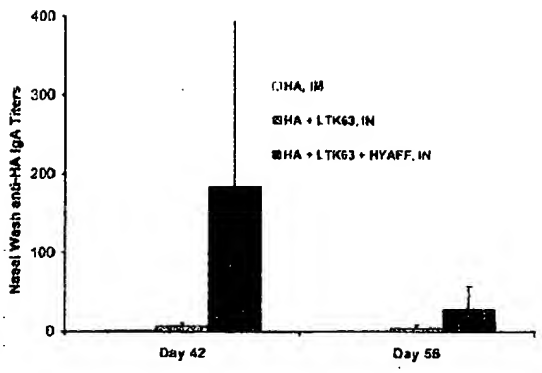

Fig. 6. Anti-HA nasal IgA antibody titers in three groups of micro-pigs (n=4) immunized with either HA alone IM, HA+LTK63 IN or HA+LTK63+HYAFF IN. The graph represents the geometric mean titers±S.E.M. for each group at days 42 and 56. The HYAFF+LTK63+HA formulation was significantly better than the HA (IM) and HA+LTK63 (IN) at days 42 and 56 ($P<0.05$).

formulation administration, a situation that does not accurately reflect an approach that would prove acceptable in humans. Hence it was considered necessary to further evaluate the bioadhesive formulations in more rigorous studies in larger animal models. In the mouse study, the bioadhesive microspheres alone did not provide an adjuvant effect and the presence of the LT mutant was necessary to achieve potent immune responses following intranasal administration. Therefore, the bioadhesive formulation alone with HA, without the addition of LT mutants, was not evaluated further. The poor response to the bioadhesive formulation alone delivered mucosally is consistent with observations from an earlier study, in which only a marginal enhancement was seen with mucosal immunization with bioadhesive microspheres and mucosal delivery was not comparable with IM immunization [31]. In contrast, an alternative bioadhesive polymer, chitosan, which is a widely used pharmaceutical excipient [32], has shown encouraging results following IN immunization in a small animal model [33]. Nevertheless, chitosan and related molecules have previously been described as potent adjuvants or immunomodulatory compounds following parenteral immunization [34]. Therefore, the adjuvant effect with IN chitosan may not come solely from the bioadhesive properties of this polymer. It remains to be seen if simple 'bioadhesion' is enough to impart a potent adjuvant effect following mucosal delivery, but our data would seem to indicate that inclusion of an adjuvant active molecule may also be necessary to induce a potent response.

In the rabbit studies, the observations from the mouse model were extended to a larger animal and the second LT mutant, LTR72 was also evaluated. In previous studies, LTR72 was shown to be a more effective mucosal adjuvant in mice than LTK63 for a model protein [15]. In addition, studies in mice with HA have shown that IN HA+LTR72 was capable of

Table 2
Hemagglutination-inhibition (HI) titers and serum IgG titers at day 42 from individual animals in three groups of micro-pigs immunized with either HA alone IM, HA+LTK63 IN or HA+LTK63+HYAFF IN

Formulation	Route	Serum IgG ELISA titers (Day 42)	Hemagglutination inhibition titers (Day 42)
HA alone	IM	137	80
		2668	640
		273	160
		589	160
HA+LTK63	IN	97	40
		1499	1280
		86	320
		984	640
HA+LTK63+HYAFF	IN	1908	1280
		889	320
		1764	640
		485	2560

inducing more potent serum immune responses than IM immunization with HA at similar dose [9]. Therefore, LTR72 was evaluated in the first rabbit study, which showed a clear demonstration of the benefit of delivering the HA-LTR72 combination in a bioadhesive microsphere delivery system. This study also served to confirm the significant potential of LTR72 as an IN adjuvant for HA, both with and without the HYAFF. Since the LTR72 bioadhesive formulation was very potent in rabbits, we also decided to evaluate the LTK63 bioadhesive formulation that had previously performed so well in the mouse study. In addition, we also decided to compare the IN bioadhesive formulation with the traditional approach to immunization, using HA alone by the IM route. In a small study, involving two groups of rabbits, the HYAFF-HA-LTK63 combination formulation was compared to IM immunization with a similar dose of HA. Both the serum IgG and the serum HI titers induced by IN immunization in this study were comparable, or greater than those induced by IM immunization. These observations encouraged us to continue with this formulation approach into a larger animal model, the pig. The omnivorous pig has all the components of the ring of lymphoid tissue, the Waldeyer's ring, which are found in humans and therefore represents a good animal model to evaluate intranasal immunization approaches [27].

For the pig study, we continued to use the LTK63 adjuvant, since it is completely non-toxic both in vitro and in vivo [16] and therefore, is the preferred candidate for subsequent human studies. In the pig study, we undertook a rigorous evaluation of the combination bioadhesive formulation and compared the responses obtained IN, with those obtained after IM immunization with the same dose of HA. Very encouragingly, IN immunization with soluble HA+LTK63 induced comparable serum IgG antibody responses to IM immunization with an equivalent dose of HA. This observation further confirmed the significant potential of LTK63 as a mucosal adjuvant and extended our earlier observations in mice [9,14–17] to a larger animal. Furthermore, the bioadhesive HYAFF-HA-LTK63 formulation induced a significantly enhanced serum IgG antibody response in comparison to IM immunization with HA. The bioadhesive formulation also induced a significantly enhanced nasal wash IgA antibody response. In addition, despite significant variability amongst individual pigs, there was a clear trend for the bioadhesive formulation to also induce enhanced HI titers over IM immunization.

The observations from these studies are notable in several ways. Firstly, they serve to illustrate the potency of the LT mutants as IN adjuvants for HA in three animal models. Studies already published [9,14–17] and many unpublished observations (O'Hagan et al., in press) have shown that these mutants are potent mucosal adjuvants for a wide range of immunogens, to include recombinant proteins, protein polysaccharide conjugates, peptides and DNA, when delivered by several different routes. In a previous study, we compared a range of different antigen delivery systems and adjuvants, including microparticles and Iscom's, for IN delivery of a recombinant protein and showed that LTK63 was the most potent adjuvant for induction of serum immunity [16]. In addition, the results described here show that the potency of the HA+LT mutant combination can be enhanced by formulation into a bioadhesive microsphere delivery system. IN immunization with the bioadhesive microsphere formulation in pigs induced a significantly enhanced serum immune response in comparison to traditional IM immunization. Since several flu vaccines are already commercially available, if they are eventually to be replaced by new vaccines administered by the IN route, then the new vaccine must induce at least a similar level of serum immunity. This was achieved in the current studies in both rabbits and pigs. In addition, in the pig study, the IN approach also induced a significant IgA response in the nasal cavity, which might help to protect against initial infection. Although not evaluated in the current studies, IN immunization with LT mutants has previously been shown to induce potent CTL responses, which should also help with viral clearance mechanisms ([17] and unpublished data). It was also notable that in the current studies, for easy administration to all animal models, the microsphere formulations were used as suspensions in saline. The bioadhesive properties would be expected to be enhanced if the formulations were administered as dry powders, and this may have increased their potency further.

Although extensively investigated for many years, the mechanism of action of most vaccine adjuvants, including bacterial toxins remains poorly defined [35], although the toxins have been shown to induce a wide range of potent changes in immune cells [36]. Nevertheless, it appears likely that mutant toxins such as LTK63 may exert some of their adjuvant effects intracellularly due to their interaction with vesicular transport systems [36]. However, extensive in vitro work both on the adjuvant and on the delivery system will be required to accurately determine the mechanism of action of the combined formulation. The bioadhesive microspheres may contribute to the immune response obtained due to one or more of the following reasons; (a) increased duration of retention in the nasal cavity, (b) greater interaction with the epithelium, (c) enhanced absorption, or (d) sustained release from the microspheres. Each of these effects may act upon the antigen, the adjuvant, or both. Although further studies are necessary to determine the mechanism of action of the formulation, it is notable that the microspheres alone were ineffective and the presence of a mucosal adjuvant was necessary for potent responses. It is planned that the bioadhesive formulations described in the current studies will be evaluated in human clinical trials in the near future.

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